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Original Article

Oxidative Stress Effect on the Activation of Hepatic Stellate Cells

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Collagen is the most excessive extracellular matrix protein in hepatic fibrosis. Activated, but not quiescent, hepatic stellate cells (HSCs) have a high level of collagen and α smooth muscle actin (α SMA) expression. HSCs play a key role in the pathogenesis of hepatic fibrosis. We analyzed a mechanism leading to HSC activation by evaluating the role of oxidative stress and the expression of NFkB. *In vitro* study HSCs were proliferated (PCNA:2% vs 68%) and activated (α SMA: 5% vs 78%) by ascorbate/$FeSO_4$, and HSCs activated by type I collagen were blocked (PCNA: 97% vs 4%, α SMA: 86% vs 9%) by α-tocopherol. *In vivo* study means of α SMA positive cells in liver at 400 $\times$ HPF were 48.3 $\pm$ 5.2 and 15.2 $\pm$ 1.8 and [^{3}H]thymidine uptake of HSC was 529.2 $\pm$ 284.8 cpm and 223.0 $\pm$ 86.3 cpm in control and α-tocopherol treated group respectively at 32 hours after CCl_4 injection. Nuclear extracts from activated, but not from quiescent, HSCs formed a complex with the NFkB cognate oligonucleotides and α-tocopherol inhibited this bindings. This study indicates that oxidative stress plays an essential role through the induction of NFkB on HSC activation.

Key Words: Hepatic stellate cell, oxidative stress, NFkB

INTRODUCTION

Excessive production of extracellular matrix (ECM), especially collagen, in the liver can produce hepatic fibrosis, and the repeated hepatic fibrosis by various etiologies may result in liver cirrhosis. Liver cirrhosis is the irreversible and terminal state of chronic liver diseases and one of the major causes of death in Korea. To block progress to liver cirrhosis, many different studies, including virological and immunological studies have been undertaken. Recently studies upon hepatic fibrogenesis have progressed due to the development of molecular biology.

Hepatic stellate cells (HSC) play a key role in the pathogenesis of hepatic fibrosis by producing ECM,[1-4] the extent of which is dependent on the proliferation and activation of HSCs.[5-8] Therefore, the inhibition of HSC activation is viewed as one of the main ways to block the progress of hepatic fibrosis.

Many kinds of factors, such as acetaldehyde,[9] ascorbic acid,[10] TGF-β[8,11,12] and CCl_4[5,13,14] have been reported to activate HSCs and stimulate collagen gene expression. Recently oxidative stress has been highlighted as one of the main causes of tissue damage. On the other hand, some kinds of cells, such as fibroblasts[10,15] are activated by oxidative stress and inhibited by antioxidant. HSC perhaps have similar characteristics because activated HSCs show a myofibroblast like feature which show α smooth muscle actin (α SMA), cytoskeletal element in cytoplasm. NFkB is an important transcriptional factor related to oxidative stress. We analyzed the effect of oxidative stress and of α-tocopherol as antioxidant, and the expression of NFkB on HSCs *in vitro* and *in vivo*.

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MATERIALS AND METHODS

In vitro study

Hepatic stellate cells (HSC) were isolated from male Sprague Dawley rats (500-600 g). Rat livers were perfused with Hank's buffer containing 0.02% pronase (Boehringer Mannheim, Mannheim, Germany) and 0.015% collagenase (Boehringer Mannheim, Mannheim, Germany) and then redigested with Hank's buffer containing 0.02% pronase in a 37℃ shaking water bath for 15 minutes, as described previously.[3] After several washes with Schwartz buffer, the cells were mixed with 9.5 ml of Hank's buffer containing 0.3% bovine serum albumin and 8 ml of 28.7% Nycodenz (Sigma Chemical Co, St Louis, MO, U.S.A.). 6 ml of Hank's buffer containing 0.3% bovine serum albumin was layered on top of the cell mixture. After centrifugation (1000 g, 4℃, 20 min) HSCs were isolated from the floating band, and then washed with serum free DME (GIBCO BRL Life Technologies, Grand Island, NY, U.S.A.), and cultured in 5% CO_2, 95% air using DME media containing penicillin G 100 U/ml, streptomycin sulfate 100 μg/ml (GIBCO BRL Life Technologies, Grand Island, NY, U.S.A.) and 10% fetal calf serum. $2-3 \times 10^6$ cells were plated on 60 mm dishes coated with EHS (Englebreth Holm Swarm, Matrigel, Collaborative Biochemical Products, Bedford, MA) or type I collagen. HSCs cultured in EHS coated dishes were treated with ascorbate/ $FeSO_4$ (200 μM/50 μM, Sigma Chemical Co, St Louis, MO, U.S.A.) and those cultured in type I collagen coated dishes were treated with α-tocopherol (50 μM, Sigma Chemical Co, St Louis, MO, U.S.A.) every 48 hours for 6 days.

In vivo study

Each sprague Dawley rat (100-150 g) received a single intraperitoneal injection of CCl_4 in mineral oil (1 : 3, vol/vol) at a dose of 2 ml/kg. The control group received CCl_4 only, the other group received CCl_4 with an intraperitoneal injection of α-tocopherol (200 mg/kg) daily from 2 days before CCl_4 injection until sacrifice. 30 μCi [^{3}H] thymidine (Amersham Pharmacia Biotech, Buckinghamshire, England) was injected intraperitoneally 4 hours before sacrifice. At 32 hours after the CCl_4 injection, 10 animals of each group were sacrificed, and their livers removed. HSCs were isolated by the nonperfusion method. Briefly, after mincing, livers were digested with Hank's buffer containing 0.5% pronase (Boehringer Mannheim, Mannheim, Germany) and 0.05% collagenase (Boehringer Mannheim, Mannheim, Germany) in a 37℃ shaking water bath for 30 minutes. The procedure used after digestion was the same as that for the perfusion method of the *in vitro* study.

Immunohistochemistry

In vitro study HSCs were fixed with acetone: methanol (1 : 1) at -20℃ for 20 minutes. *In vivo* study paraffin embedded liver tissues were deparaffinized with xylene and ethanol. After blocking with normal goat serum, both samples were treated with primary antibodies (HSC: α SMA (Sigma Chemical Co, St Louis, MO, U.S.A.) and PCNA (Sigma Chemical Co, St Louis, MO, U.S.A.), liver: α SMA). After treating with biotinylated secondary antibodies and avidin-biotin alkaline phosphatase complex, NBT-BCIP was used as a cytochrome and counterstained with 1% fast green *in vitro* study. Two experienced observers counted at least 1000 cells per experimental point and immunostained positive cells were expressed as a percentage of total cells *in vitro* study. 10 portions of each liver tissue were examined at 400 × high power field (HPF). Immunostained positive cells were expressed as a mean value of 400 × HPF *in vivo* study.

[^{3}H]thymidine uptake

In vivo study isolated HSC were treated with TRIzol reagent (GIBCO BRL Life Technologies, Grand Island, NY, U.S.A.) and chloroform. After centrifugation (12000 g, 4℃, 15 min) the upper phase was removed and 100% ethanol was mixed with the lower phase to obtain the DNA pellet. After several washings with 0.1 M sodium citrate and 75% ethanol, the DNA pellets were dissolved in 8 mM NaOH. A β counter was used to count [^{3}H]thymidine incorporation into 10 μg of DNA.

Electrophoretic Mobility Shift Assay (EMSA)

HSCs were homogenized with 1ml of 5% citric acid containing 0.5% NP40, 10 mM NaF and 10 mM Na pyrophosphate. Homogenized cells were layered on top of a solution containing 30% sucrose and 1% citric acid. After centrifugation (4000 g, 4℃, 30 min) nuclear pellets were obtained and stored at -70℃. After lysis, 3 μg of nuclei at OD 260 nm were mixed with ^{32}P-labeled NFkB binding oligonucleotides (5, GGG GAC TTT CCC 3′), band shift buffer and polydIdC and then electrophoresed in 6% polyacrylamide gel. After electrophoresis, the gel was fixed with 10% acetic acid and 20% ethanol and transfered to paper with a gel dryer. After attaching the film to paper overnight, films were developed and shifted bands examined.

Statistical Analysis

In vitro study the results were expressed as the means of triplicates of immunostain. Student t test was used, and a p value of < 0.05 was taken as significant. *In vivo* study results were expressed as means of 10 animals. The Mann-Whitney U test was used to evaluate the difference between the control and α-tocopherol treated groups.

RESULTS

In vitro study

HSCs cultured on EHS matrix showed the quiscent state (α SMA: 5%, PCNA: 2%) (Fig. 1-3, and 4) because EHS matrix, which has a basement membrane composition containing type IV collagen and laminin, inhibited the activation and proliferation of HSCs. When HSCs cultured on EHS matrix were treated with ascorbate/$FeSO_4$,

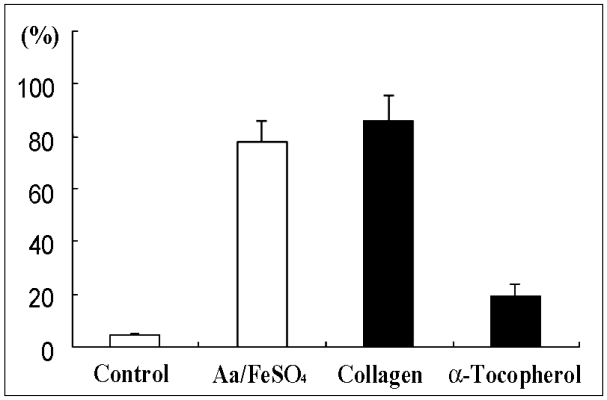

Fig. 1. α smooth muscle actin (α SMA) expression was detected by immunohistochemistry in hepatic stellate cells. Cells were cultured on EHS matrix (open bars: control, ascorbate/$FeSO_4$ [200 μM/50 μM]) and type I collagen matrix (closed bars: collagen, α-tocopherol [50 μM]). Values represent the percentage of cells positive for α SMA: $P<0.05$ for ascorbate/$FeSO_4$ and collagen. The SEM was < 30% of the mean for all conditions.

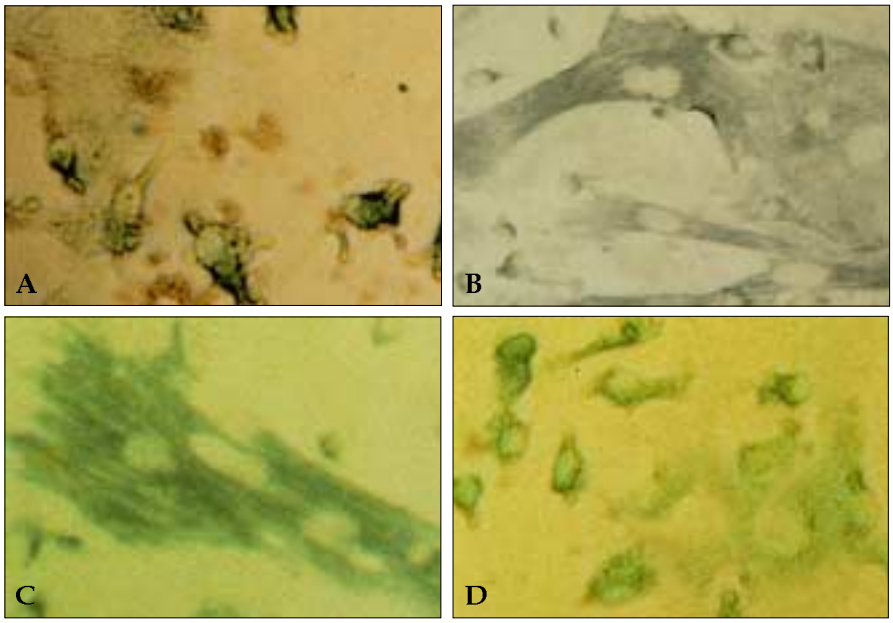

Fig. 2. The cytoplasmic expression of α smooth muscle actin (α SMA) was detected by immunohistochemistry in cultured hepatic stellate cells using alkaline phosphatase, while fast green counterstained cells. α SMA immunohistochemistry is shown for control (A), ascorbate/$FeSO_4$ (B), type I collagen matrix (C) and α-tocopherol (D). ×100.

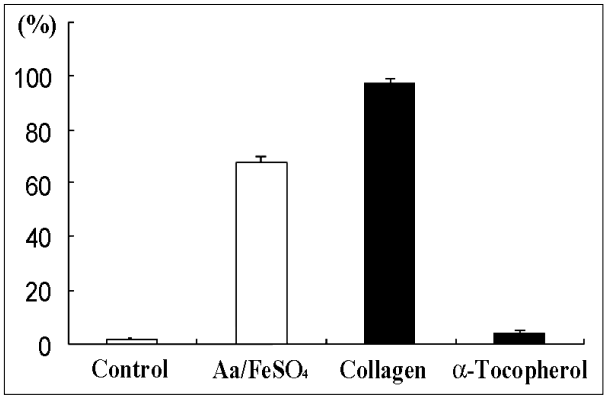

Fig. 3. The nuclear expression of PCNA was detected by immunohistochemistry in hepatic stellate cells. Cells were cultured on EHS matrix (open bars: control, ascorbate/$FeSO_4$ [200 μM/50 μM]) and type I collagen matrix (closed bars: collagen, α-tocopherol [50 μM]). Values represent the percentage of cells positive for PCNA: $P<0.05$ for ascorbate/FeSO4 and collagen. The SEM was <30% of the mean for all conditions.

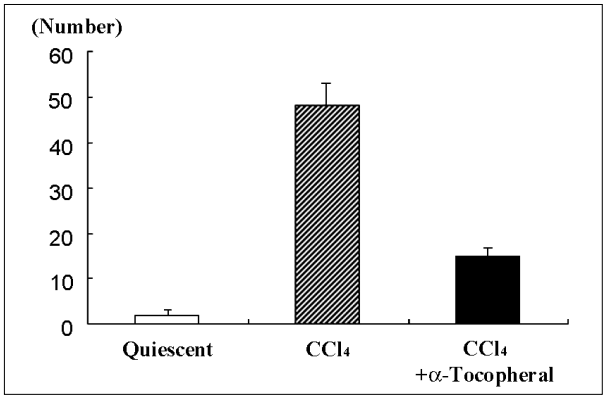

Fig. 5. Means of α smooth muscle actin (α SMA) positive hepatic stellate cells in liver at 400× HPF were detected by immunohistochemistry. Quiescent means normal livers which were removed before CCl_4 injection. Hatched bars showed CCl_4 only (Group I) and closed bars showed CCl_4+ α-tocopherol (Group II). $P<0.05$ for CCl_4. The SEM was < 30% of the mean for all conditions.

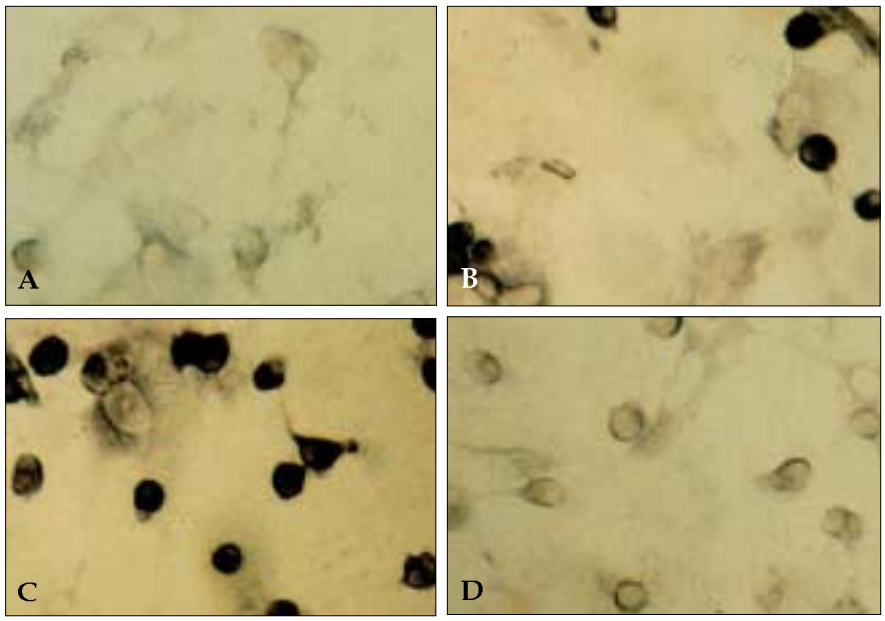

Fig. 4. The nuclear expression of PCNA was detected by immunohistochemistry in cultured hepatic stellate cells using alkaline phosphatase, while fast green counterstained cells. PCNA immunohistochemistry is shown for control (A), ascorbate/$FeSO_4$ (B), type I collagen matrix (C) and α- tocopherol (D). × 100

which generate free radicals and induces oxidative stress, HSCs were activated (α SMA: 78%) (Fig. 1, and 2) and proliferated (PCNA: 68%) (Fig. 3, and 4) significantly compared to the control. HSCs cultured on type I collagen, which is the main extracellular matrix in which hepatic fibrogenesis occurs, were activated (α SMA: 86%) (Fig. 1, and 2) and proliferated (PCNA: 97%) (Fig. 3, and 4). When HSCs cultured on type I collagen were treated with α-tocopherol, a well known antioxidant, activation and proliferation were significantly inhibited (α SMA: 19%, PCNA: 4%) (Fig. 1-3, and 4).

In vivo study

The means of the α SMA positive cells at 400 × HPF were 48.3 ± 5.2 and 15.2 ± 1.8 (Fig. 5 and 6) in the control and α-tocopherol treated groups respectively at 32 hours after CCl_4 injection. Activation of HSCs was significantly inhibited in the α-tocopherol treated group. The [^{3}H]thymi-

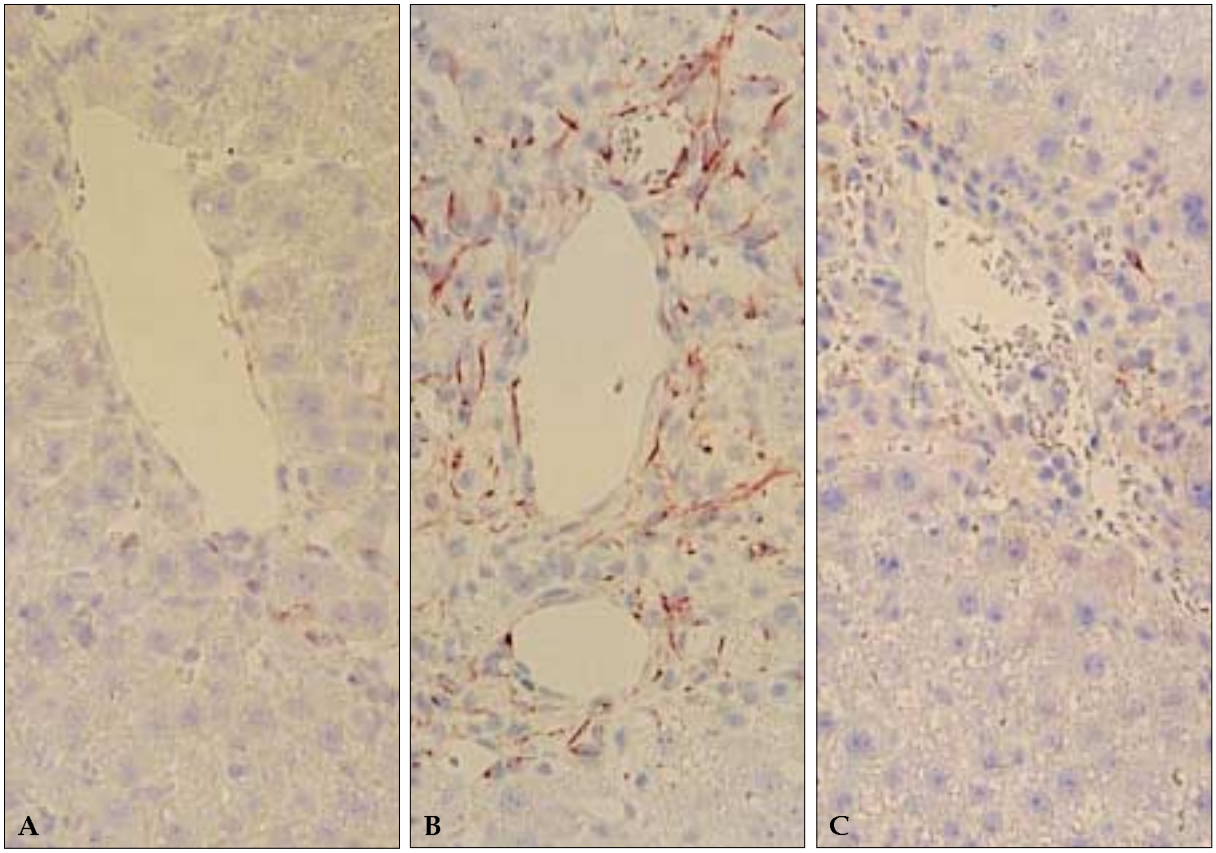

Fig. 6. α Smooth muscle actin (α SMA) expression of hepatic stellate cells was detected by immunohistochemistry in liver using alkaline phosphatase. α SMA immunohistochemistry is shown for control (A), CCl_4 only (B) and CCl_4 + α -tocopherol (C). ×200

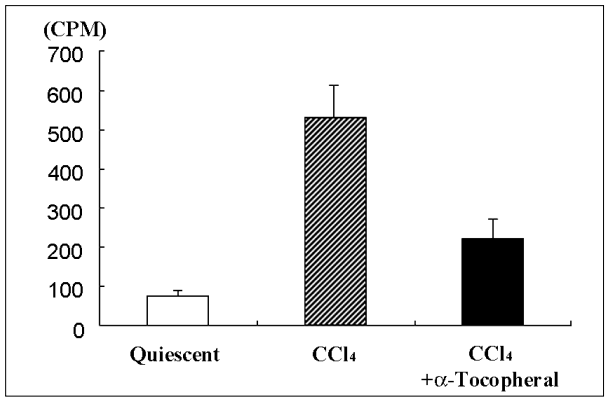

Fig. 7. Means of [^{3}H]thymidine uptake of hepatic stellate cells were detected by β counter. Quiescent means normal livers which were removed before CCl_4 injection. Hatched bars showed CCl_4 only (Group I) and closed bars showed CCl_4 + α-tocopherol (Group II). $P < 0.05$ for CCl_4. The SEM was <30% of the mean for all conditions.

dine uptake study correspondingly showed 76.4 ± 14.7 cpm (Fig. 7) in the HSCs of quiscent state prior to CCl_4 injection. At 32 hours after the CCl_4 injection, [^{3}H]thymidine uptake study showed 529.2 ± 84.8 cpm and 223.0 ± 46.3 cpm (Fig. 7) in the control and α-tocopherol treated groups respectively. Proliferation of HSCs was significantly inhibited in the α-tocopherol treated group.

Electrophoretic Mobility Shift Assay (EMSA)

The binding of HSC nuclear extracts to NFkB cognate oligonucleotides was absent or low (Fig. 8, and 9, lane 2) in the quiescent state, but increased significantly following HSC activation with type I collagen (Fig. 8, lane 3) and CCl_4 (Fig. 9, lane 3). Because activation of HSCs was inhibited by α- tocopherol, the binding of HSC nuclear extracts to NFkB cognate oligonucleotides were low (Fig. 8, and 9, lane 4). Non-labeled NFkB oligonucleotides inhibited competitively the binding of ^{32}P-labeled NFkB oligonucleotides to nuclear extracts from activated HSCs (Fig. 8, and 9, lane 5).

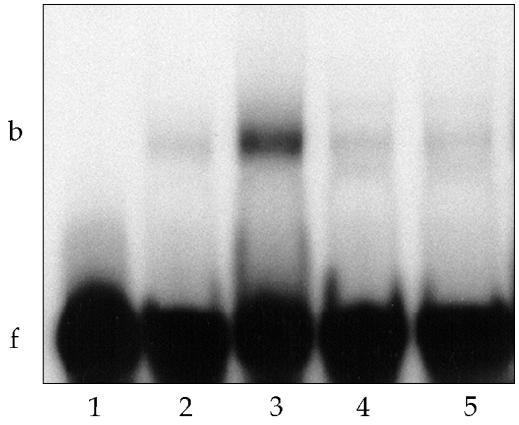

Fig. 8. Electrophoretic mobility shift assay showed increased NFkB binding activity in hepatic stellate cells activated by type I collagen (lane 3) and NFkB binding activity was inhibited by α-tocopherol (lane 4). Lane 2 was incubated with nuclear extract of quiescent hepatic stellate cells. Lane 1 was incubated without nuclear extracts. Non-labeled NFkB oligonucleotides competitively inhibited NFkB binding activity (lane 5). The position of the free(f) and bound(b) DNA are indicated.

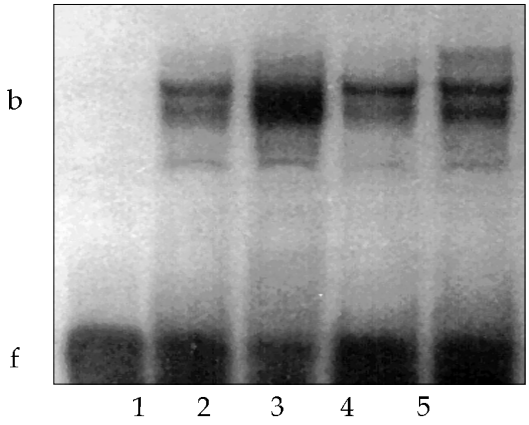

Fig. 9. Electrophoretic mobility shift assay showed increased NFkB binding activity in hepatic stellate cells activated by CCl_4 (lane 3) and NFkB binding activity was inhibited by α-tocopherol (lane 4). Lane 2 was incubated with nuclear extract of quiescent hepatic stellate cells. Lane 1 was incubated without nuclear extracts. Non-labeled NFkB oligonucleotides competitively partially inhibited NFkB binding activity (lane 5). The position of the free(f) and bound(b) DNA are indicated.

DISCUSSION

Whenever, an organ is injured by various etiologies, a repair process is initiated to recover to the normal condition. In acute injury each organ is recovered completely without scarring change or complication, but in chronic injury a large portion of the organ become fibrosis due to the excessive production of extracellular matrix (ECM). In chronic liver injury hepatic stellate cells (HSC)[16,17] are activated by cytokines that originate from damaged hepatocytes and Kupffer cells which phagocytose damaged hepatocytes. Activated HSCs express α smooth muscle actin (α SMA), a cytoskeletal element in the cytoplasm and produce ECM as collagen. Cytokines, especially TGFβ, activate HSCs by the autocrine effect and induce apoptosis of hepatocytes.

In general oxygen free radicals are associated with cell damage by lipid peroxidation, depletion of glutathion and thiol compounds, binding with macromolecules, and producing an imbalance of intracellular free calcium, and DNA fragmentation. Oxidative stress is related to liver diseases like alcoholic liver disease,[18,19] heavy metal induced liver injury, ischemia-reperfusion liver injury,[20,21] and chronic hepatitis C.[22] We already reported the relation between lipid peroxidation and disease activity in chronic liver disease, type B.[23] Therefore, oxidative stress can be considered to be one of the major causes of liver damage.[24] On the other hand oxidative stress is also related to hepatic fibrogenesis by the stimulation of collagen gene expression associated with lipid peroxidation in hepatocellular injury.[3] Ascorbic acid acts as an electron donor to reduce Fe^{3+} to Fe^{2+} which further reacts with O_2 to generate superoxide, hydrogen peroxide and hydroxyl radical in turn.[10] These oxygen free radicals can damage the cell membrane, protein and DNA, but some kinds of cells, such as fibroblasts are activated rather than damaged by oxidative stress. We used ascorbic acid and $FeSO_4$ to generate oxygen free radicals *in vitro*. Our results show that HSCs were activated by ascorbic acid and $FeSO_4$ induced oxidative stress, nevertheless, pretreated EHS inhibited the activation of HSC.

CCl_4 is a representative toxic material, that injures the liver by forming oxygen free radicals, such as trichloromethyl peroxy radical, which has been well studies in acute and chronic oxidative stress liver injury models.[5,13,14] Because we had preliminary data that liver injury peaked at 32 hours after a single CCl_4 injection, we isolated HSCs at this time. HSCs were activated in this acute CCl_4 induced liver injury. Because HSC

were activated by oxidative stress *in vitro* and *in vivo* study, oxidative stress is believed to be an important factor and antioxidants may inhibit the activation of HSCs.

In normal liver, space of Disse which contain resting state HSCs, consists of type IV collagen and laminin.[25,26] After the type IV collagen of the basement membrane is disrupted by type IV collagenase produced by HSCs in chronic liver injury, the HSC become more activated and show increased response to growth factors. This results in their production of type I collagen which further activates the HSCs.[8] *In vitro* study α-tocopherol inhibited the activation of HSCs even type I collagen activate HSCs. This result demonstrates that type I collagen activate HSC through oxidative stress. *In vivo* study α-tocopherol also inhibited CCl_4 induced HSC activation and proliferation. α-tocopherol has been widely used as a vitamin supplement and an antioxidant, and now also shows the potential to inhibit HSC activation, by means of its antioxidant properties by this study.

Pentoxifylline is a nonspecific inhibitor of cyclic nucleotide phosphodiesterase and inhibits collagen gene expression in dermal fibroblasts. We previously reported that the inhibition of HSC activation by pentoxifylline resulted from the blocking of the oxidative stress cascade within the HSCs, rather than from its phosphodiesterase inhibitory activity.[27]

NFkB was first identified as a transcriptional factor that activates intron enhancer of immunoglobulin kappa light chain during B lymphocyte development.[28] In the liver NFkB has both beneficial and harmful effects, because it promotes liver regeneration and inhibits apoptosis,[29] but it also increases the transcription of the intercellular adhesion molecules (ICAM)-1 and interleukin-6, which contribute to hepatic inflammation in activated HSCs during hepatic fibrogenesis.[30] Some reports[31,32] have shown that NFkB DNA binding activity was elevated, and the supershift was shown by using p50 and p65 antibodies in activated HSCs *in vitro*, but another study[33] showed that cultured HSCs were proliferated as was the control, nevertheless, NFkB activity was inhibited by using an adenovirus expressing an IkB dominant negative protein. So the role of NFkB in activated HSCs is still contriversial, moreover there has been no study to date of activated HSCs *in vivo*. Because NFkB plays important roles in the regulation of cell growth and function, and oxidative stress induces NFkB activity,[34-36] we analyzed the potential role of NFkB regulation in HSC activation *in vitro* and *in vivo*. Our results show that HSC activation was associated with the activation of NFkB as detected by EMSA *in vitro* and *in vivo* study. *In vitro* study α-tocopherol blocked the activation of NFkB induced by type I collagen, indicating that type I collagen affects NFkB activation through oxidative stress. *In vivo* study α-tocopherol also blocked the activation of NFkB induced by CCl_4. These findings suggest that NFkB is the molecular mediator of oxidative stress on HSC activation.

Therefore oxidative stress is both sufficient and indispensable for the activation of HSCs *in vitro* and *in vivo*, through the induction of NFkB, moreover antioxidants such as α-tocopherol can block HSC activation and hepatic fibrogenesis.

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